High-Speed Countercurrent Chromatography

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Introduction

Countercurrent chromatography (CCC) is essentially a form of liquid–liquid partition chromatography. Its unique feature among other chromatographic systems is derived from the fact that the method uses no solid support and the stationary phase is retained in the column with the aid of gravity or centrifugal force. The method has been termed after two classic partition techniques – countercurrent distribution and liquid chromatography.

A great advance in the CCC technology was made with the discovery of a new hydrodynamic phenomenon in a rotating coiled tube, which provided the basis for developing a highly efficient CCC system called high-speed CCC (HSCCC). In the last decade, types XL, XLL, XLLL and L cross-axis coil planet centrifuges (CPCs) have been developed to perform CCC with highly viscous polar solvent systems, such as polyethylene glycol (PEG) potassium phosphate, PEG dextran aqueous–aqueous two-phase systems. The absence of a solid support eliminates various complications that might arise from this in conventional chromatographic systems and the CCC has the ability to preserve the functional and enzymatic activity of proteins.

Apparatus

The cross-axis coil CPCs, which include types X and L and their hybrids (see Figure 3 in the article on Countercurrent chromatography), are used for protein separation. These modified versions of the HSCCC centrifuge have a unique feature among the CPC systems in that the system provides reliable retention of the stationary phase for viscous polymer-phase systems. **Figure 1** shows five different types of cross-axis CPCs. A series of studies has shown that the stationary-phase retention is enhanced by laterally shifting the position of the coil holder along the holder shaft, apparently due to the asymmetry of the laterally acting force field between the upper and the lower halves of the rotating coil. The degree of the lateral shift of the coil holder is conventionally expressed by L/R, where L is the distance from the middle point of the rotary shaft to the coil holder and R is the distance from the centrifuge axis to the holder axis. Types XL, XLL, XLLL and L have been effectively used for protein separations with various polymer-phase systems. For example, the polymer-phase system composed of PEG and potassium phosphate has a relatively large difference in density between the two phases, so it can be retained in XL-XLL column positions which provide efficient mixing of the two phases. On the other hand, the viscous polymer-phase system composed of PEG and dextran has an extremely low interfacial tension and small density differences between the two phases so that they tend to emulsify under vigorous mixing. Therefore, the type XLLL or L column position, that provides less efficient mixing under a strong centrifugal force field, is required to achieve satisfactory retention of the stationary phase for this polymer-phase system.

Figure 2 shows the XLLL cross-axis CPC ($L/R = 3.5$) equipped with a pair of multilayer coil separation columns. Each column consists of 2.6 mm i.d. polytetrafluoroethylene (PTFE) tubing wound on to a coil holder hub, forming multiple layers of left-handed coils. **Table 1** lists various CPC models that have been used for the preparative separation of proteins, together with various parameters, including the

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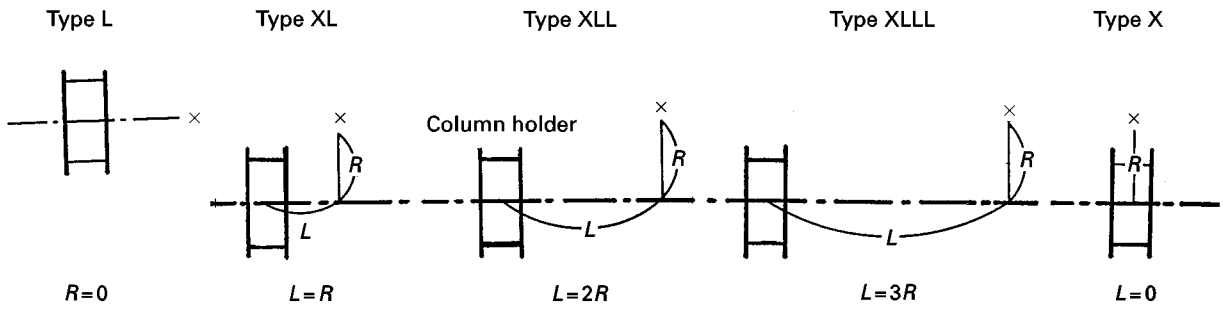

Figure 1 Orientation of the column holder on the axis of rotation in five different types of the cross-axis coil planet centrifuges. ×, axis of revolution; dashed line, axis of rotation; *L*, lateral shift; *R*, revolution radius.

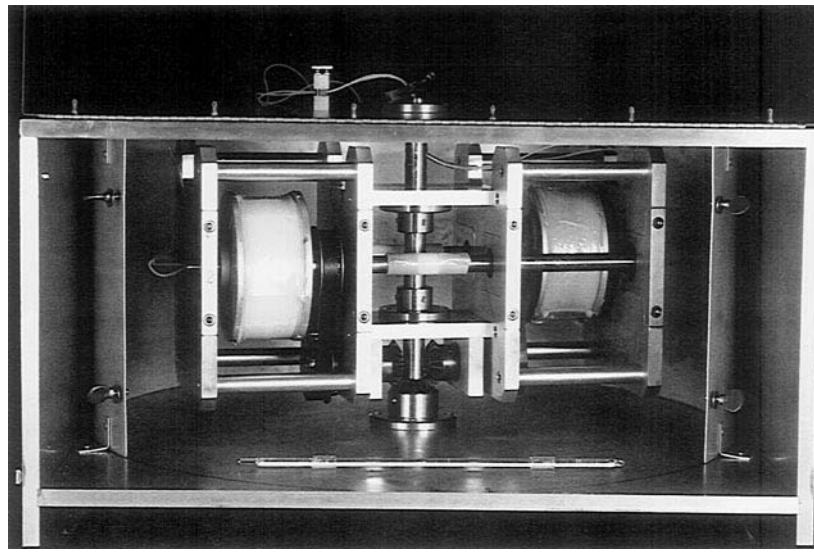

Figure 2 Type XLLL cross-axis CPC equipped with a pair of multilayer coils connected in series.

dimensions of columns and column holders, and β values of multilayer coils.

Polymer-phase Systems for Preparative Separation of Proteins

CCC utilizes a pair of immiscible solvent phases pre-equilibrated in a separatory funnel where one phase is used as the stationary phase and the other as the mobile phase. There are two typical polymer-phase systems available for protein separation: PEG dextran and PEG potassium phosphate systems.

PEG Dextran Systems

The polymer-phase system composed of PEG and dextran has a characteristic feature: small molecules are partitioned fairly evenly between the two phases, whereas macromolecules such as DNA and polynucleic acids are distributed unilaterally in one phase or the other, depending on the pH of the solvent system. Consequently, the system can be used effectively for separation of these macromolecules using pH gradient elution. The PEG dextran system forms two layers without addition of high salt concentration, which tends to be precipitated in PEG phosphate systems (see below) at high salt concentrations. On the other hand, the PEG dextran system has a serious drawback in its CCC application. At high dextran concentrations the viscosity of the lower phase increases, and the similar polarity of the two polymers reduces interfacial tension between the two phases, resulting in a high probability of emulsification. A typical PEG dextran polymer system contains 4.4% (w/w) PEG 8000, 7% (w/w) dextran T500 and 10 mmol L^{-1}

Table 1 Type of apparatus and dimensions of columns used for protein separation

x-axis CPC	*(L/R)*[a]	*Coil holder*		*Columns*				
		Diameter (cm)	*Width (cm)*	*i.d. (mm)*	*Length (m)*	*Layers*	*Capacity (mL)*	*β Values*[b]
Type XL	(1.25)	10.0	5.0	2.6	31	3	165	0.50–0.60
		15.2	5.0	2.6	11	1	60	0.76
		15.2	5.0	2.6	64	4	340	0.76–0.90
Type XLL	(2.0)	3.8	5.0	2.6	47	8	250	0.25–0.60
		7.6	5.0	2.6	53	6	280	0.50–1.00
Type XLLL	(3.5)	3.8	5.0	2.6	57	9	300	0.50–1.30
		3.4	5.0	2.6	83	12	440	0.45–1.50
Type L	(infinity)	3.6	5.0	2.6	25	5	130	0.16–0.27[c]

[a] L = Distance from the centre of the holder shaft to the coil holder; R = distance from the centrifuge axis to the holder shaft.
[b] $\beta = r/R$.
[c] $\beta = r/L$, where r is the distance from the holder axis to the coil.

Table 2 Preparation of polymer two-phase solvent systems

	Concentration (%, w/w)			pH
	PEG 1000	K_2HPO_4	KH_2PO_4	
1	12.5	12.5	0	9.0
2	12.5	9.375	3.125	7.7
3	12.5	8.33	4.17	7.3
4	16.0	12.5	0	9.2
5	16.0	10.4	2.1	8.0
6	16.0	8.33	4.17	7.3
7	16.0	6.25	6.25	6.8

potassium phosphate buffer at proper pH. This two-phase system consists of a PEG-rich upper phase and a dextran-rich lower phase.

PEG Potassium Phosphate Systems

The PEG potassium phosphate system is complementary to the PEG dextran system in that it tends to distribute low-molecular-weight compounds unilaterally in either the upper or lower phase while macromolecules such as proteins are more evenly distributed between phases. Consequently, once a suitable partition coefficient for the target protein is obtained, the system yields high-purity fractions almost free from contamination by low-molecular-weight impurities that either elute immediately after the mobile phase front or remain almost permanently in the column.

Table 2 shows the composition of seven different PEG 1000 potassium phosphate systems. The ratio of the monobasic and dibasic potassium phosphate determines the pH of the solvent system and the partition coefficient of the protein samples. In all these solvent systems, the upper layer is rich in PEG and the lower layer is rich in phosphate.

Profilin–actin Complex Purification from Crude *Acanthamoeba* Extract

Using the type L cross-axis CPC equipped with a pair of multilayer coils (130 mL capacity), profilin–actin complex has been purified directly from an *Acanthamoeba* extract with a polymer-phase system composed of 4.4% (w/w) PEG 8000, 7% (w/w) dextran T500 at pH 6.8. The lower dextran-rich phase was used as the stationary phase. The sample solution was prepared by adding the correct amounts of PEG 8000 and dextran T500 to 12.5 g of the *Acanthamoeba* crude extract to adjust the two-phase composition to that of the solvent phases used for separation. The separation was carried out by pumping the PEG-rich

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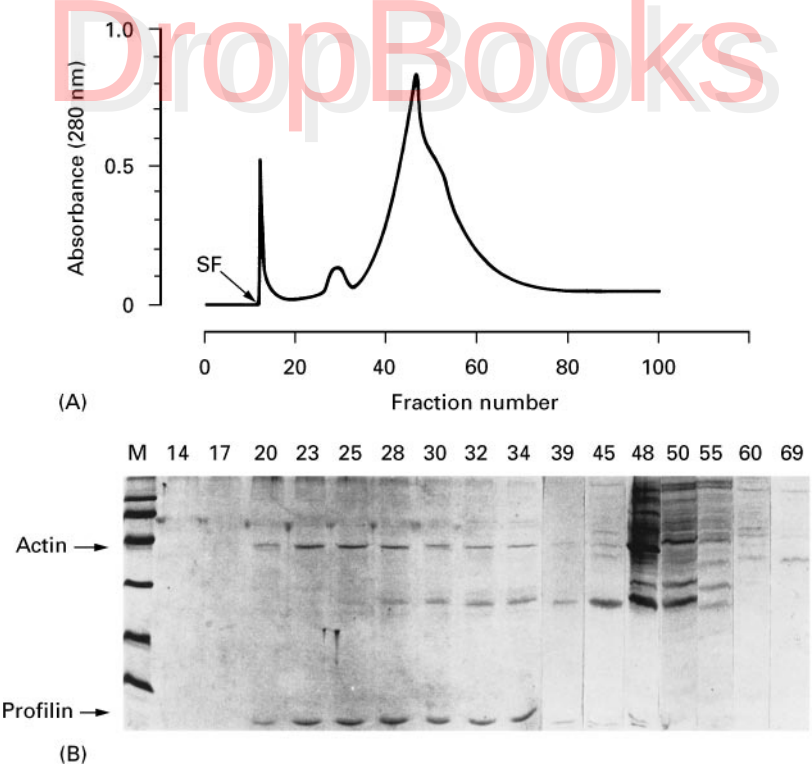

Figure 3 Purification of profilin–actin complex from *Acanthamoeba* soluble extract. Experimental conditions: column is a 2.6 mm i.d. PTFE multilayer coil × 2, $\beta = 0.16$–0.27; 130 mL capacity; sample consists of 12.5 g *Acanthamoeba* soluble extract; solvent system is 4.4% (w/w) PEG 8000, 7% (w/w) dextran T500 in a 10 mmol L^{-1} potassium phosphate buffer at pH 6.8; mobile phase is the upper phase; flow rate: 0.5 mL min^{-1}; revolution: 900 rpm; SF, solvent front.

upper phase into the head of the column at 0.5 mL min^{-1} under a high-revolution speed of 1000 rpm. The results are shown in **Figure 3A**. The solvent front emerged at the 14th fraction (3 mL per fraction) whereas the profilin–actin complex was eluted in fractions 20 to 28, well separated from other components. The impurities were mostly eluted later with a retention volume close to the total column capacity (around fractions 33–60), while some were also found near the solvent front (fraction 15). Identification of the profilin–actin complex was made by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), as illustrated in **Figure 3B**. The retention of the lower stationary phase was 69% of the total column capacity.

Countercurrent Chromatographic Fractionation of Lipoproteins from Human Serum

The performance of the XLL cross-axis CPC has been evaluated by the direct separation of high- and low-density lipoproteins (HDLs and LDLs) from human serum. The effects of the molecular weight of the PEG was studied with a polymer-phase system composed of 16% (w/w) PEG, 12.5% (w/w) potassium phosphate. **Figure 4** shows the chromatograms of human serum (4 mL) obtained from four solvent systems containing different molecular weight PEGs (600, 1000, 2000 and 4000).

In each experiment, the CCC column was first entirely filled with the PEG-rich upper stationary phase, and the sample solution (a mixture of 4 mL human serum and 2 mL each of upper and lower phases, to which the required amounts of PEG and potassium phosphate were added to adjust the two-phase composition) was injected through the sample port. Then, the potassium phosphate-rich lower mobile phase was eluted through the column at a flow rate of 2 mL min^{-1} while the apparatus was rotated at 500 rpm. The lipoprotein fractions obtained in the CCC were characterized using polyacrylamide gel disc electrophoresis (disc PAGE). Serum proteins in the CCC fractions were also characterized by SDS-PAGE.

In the PEG 600 system (Figure 4A), all proteins including HDLs, LDLs and serum proteins were strongly retained in the PEG-rich stationary phase and eluted together when the column was eluted in

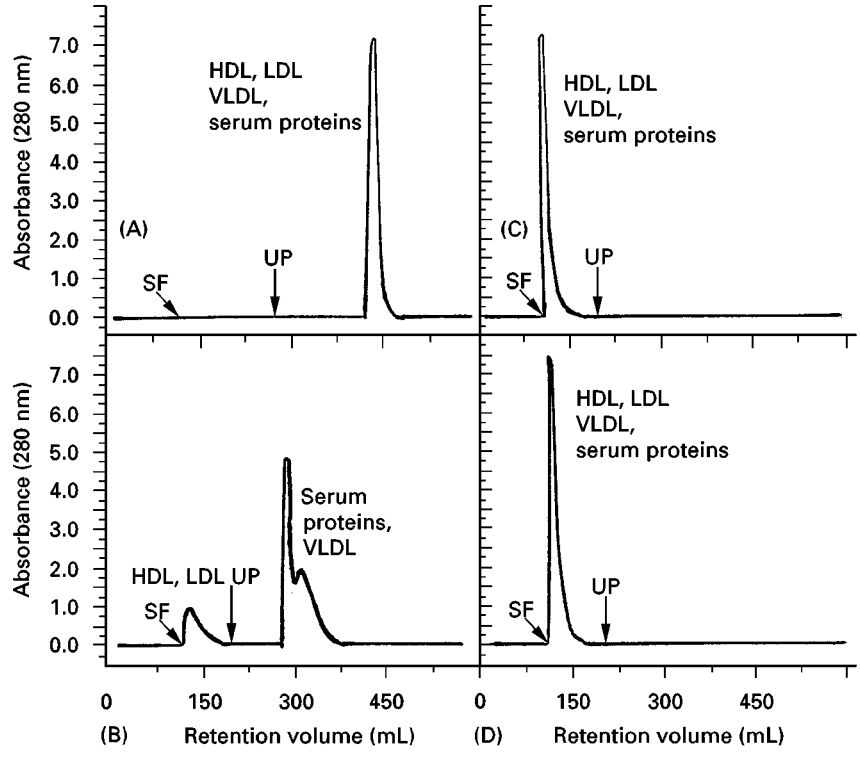

Figure 4 Countercurrent chromatographic fractionation of HDL-LDL and VLDL-serum protein fractions from human serum with four different aqueous polymer-phase systems containing (A) PEG 600; (B) PEG 1000; (C) PEG 2000; (D) PEG 4000. Experimental conditions: column is a 2.6 mm i.d. PTFE multilayer coil × 2, $\beta = 0.76$–0.90, 340 mL capacity; sample is a mixture of 4 mL volume of human serum, 2 mL of the upper and the lower phases, to which the required amounts of PEG and potassium phosphate were added to adjust the two-phase composition; solvent system consists of 16% (w/w) PEG 1000, 12.5% (w/w) K_2HPO_4 (pH 9.2); mobile phase is the lower phase; flow rate: 2.0 mL min^{-1} revolution: 500 rpm; SF, solvent front; UP, starting point of the reversed elution mode with the upper phase mobile.

a reversed elution mode with the PEG-rich upper phase. Similarly, when PEGs with molecular weights higher than 2000 were used in the solvent system, all proteins including HDLs, LDLs and serum proteins were mostly distributed to the potassium phosphate-rich lower phase and eluted together at the solvent front (Figure 4C and D). Successful separation of the combined HDL and LDL fraction was achieved with the 16% (w/w) PEG 1000, 12.5% (w/w) potassium phosphate solvent system at pH 9.2, where both HDLs and LDLs were eluted together near the solvent front, while other proteins, including very-low-density lipoproteins (VLDLs) and serum proteins were retained in the column for much longer. The separation time of these two lipoproteins was 3 h. The VLDLs were eluted by the PEG-rich upper phase in the second peak or its shoulder (Figure 4B).

These results show that both HDL-LDL and VLDL-serum protein fractions were fractionated within 3 h by CCC with a polymer-phase system composed of 16% (w/w) PEG 1000 and 12.5% (w/w) dibasic potassium phosphate at a relatively high flow rate of 2 mL min^{-1}.

Purification of HDLs, LDLs and VLDLs from Human Serum by Combined Use of CCC and Hydroxyapatite Chromatography

In the previous section, two lipoprotein fractions (HDL-LDL and VLDL-serum proteins) were obtained from human serum using a polymer-phase system by the type XL cross-axis CPC equipped with a large-capacity column (340 mL). A small-capacity column (60 mL) mounted on the same apparatus can be employed to shorten the separation time. **Figure 5** shows a chromatogram of human serum (4 mL) obtained with the cross-axis CPC using 16% (w/w) PEG 1000, 12.5% (w/w) dibasic potassium phosphate (pH 9.2). The separation was performed at 500 rpm and at a flow rate of 0.5 mL min^{-1} using the lower phase as the mobile phase. Both HDLs and LDLs were eluted together near the solvent front, while other proteins were retained in the column much longer. After collecting the HDL-LDL fraction (CCC-fr. 1), VLDLs were eluted together with serum proteins (CCC-fr. 2) by pumping the upper phase in the reverse direction. The separation was completed within 4.5 h. The lipoproteins in each peak were confirmed by agarose gel electrophoresis with Oil Red 7B staining, and the serum proteins were also detected by 10% SDS-PAGE with Coomassie Brilliant Blue protein staining.

The CCC fractions 1 (HDL-LDL) and 2 (VLDL-serum proteins) were each separately dialysed against distilled water until the concentration of the potassium phosphate was reduced to that in the starting

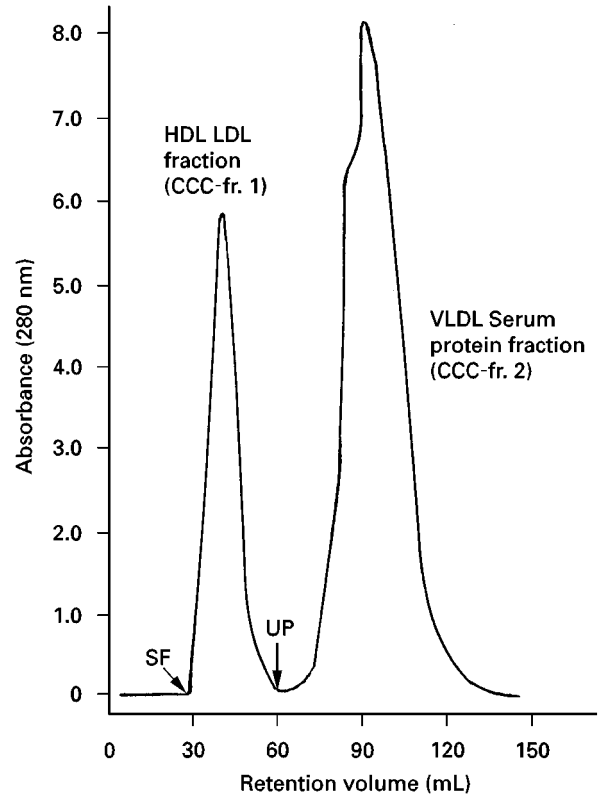

Figure 5 Countercurrent chromatographic separation of HDL-LDL and VLDL-serum protein fractions from human serum by small-capacity columns. Experimental conditions: column is a 2.6 mm i.d. PTFE multilayer coil × 2, $\beta = 0.76$, 60 mL capacity; sample is a 4 mL volume of human serum containing 0.9 g PEG 1000 and 0.7 g dibasic potassium phosphate; solvent system consists of 16% (w/w) PEG 1000, 12.5% (w/w) K_2HPO_4 (pH 9.2); mobile phase is the lower phase; flow rate: 0.5 mL min^{-1}; revolution: 500 rpm; SF, solvent front; UP, starting point of the reversed elution mode with the upper phase mobile.

buffer used for hydroxyapatite chromatography. The concentrates of both fractions were chromatographed separately on the hydroxyapatite column. **Figure 6** shows the elution profile on hydroxyapatite obtained from CCC-fr. 1. A 1.4 mL volume of the concentrate was loaded on the Bio-Gel HTP DNA-grade column (5.0 × 2.5 cm i.d.) and eluted at 1.0 mL min^{-1} with 75 and 290 mmol L^{-1} potassium phosphate buffer at pH 7.4. Two lipoprotein peaks were eluted: the first peak (HA-fr. 1) contained HDLs and the second peak (HA-fr. 2) contained LDLs.

The concentrate (1.5 mL) of CCC-fr. 2 was similarly chromatographed (**Figure 7**). The separation was performed with two-step elution with 290 and 650 mmol L^{-1} potassium phosphate buffers at pH 7.4. Most of the serum proteins, including albumin and globulins, were eluted with 290 mmol L^{-1} potassium phosphate buffer (HA-fr. 3) at pH 7.4. The VLDLs, on the other hand, were retained in the

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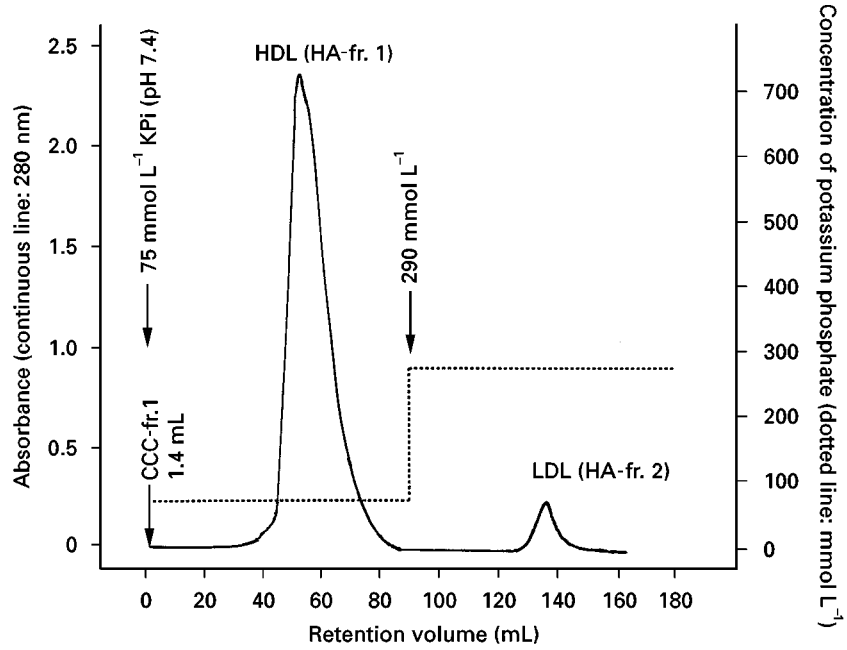

Figure 6 Stepwise elution profile of HDLs and LDLs of the CCC fractions by hydroxyapatite chromatography. Experimental conditions: column is Bio-Gel HTP DNA-grade hydroxyapatite (5.0 × 2.5 cm i.d.); eluents are 75 and 290 mmol L^{-1} potassium phosphate buffers at pH 7.4; flow rate: 1.0 mL min^{-1} sample is the 1.4 mL concentrate of HDL-LDL CCC fraction containing 13.9 mg total proteins (CCC-fr. 1).

column for much longer and were eluted with 650 mmol L^{-1} potassium phosphate buffer (HA-fr. 4). Lipoproteins in the hydroxyapatite chromatographic fractions were confirmed by agarose gel electrophoresis. The results of agarose gel electrophoresis indicated that HDLs, LDLs and VLDLs were present

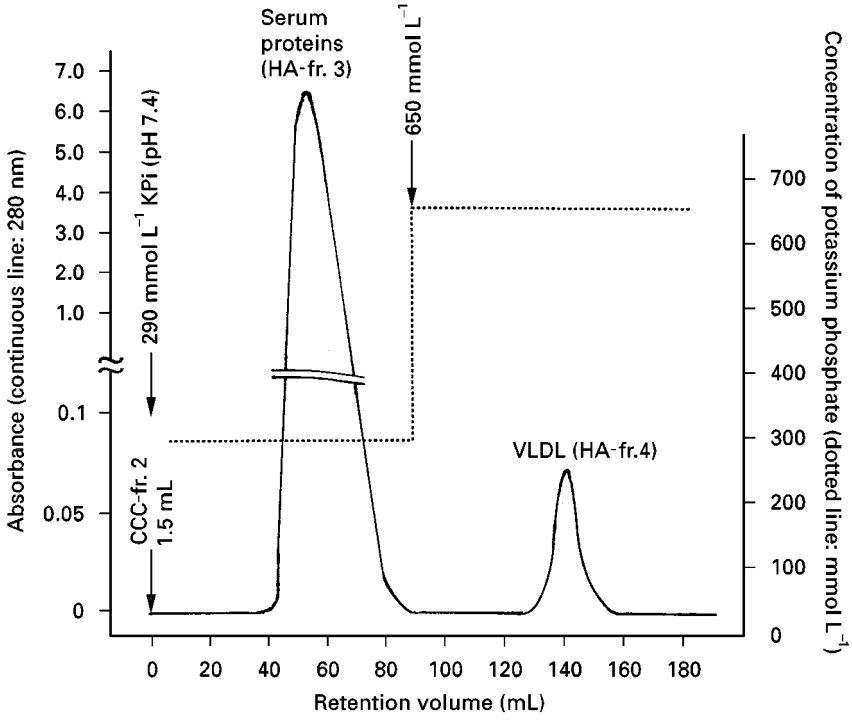

Figure 7 Stepwise elution profile of VLDL-serum proteins fraction of the CCC fractions by hydroxyapatite chromatography. Experimental conditions: column is Bio-Gel HTP DNA-grade hydroxyapatite (5.0 × 2.5 cm i.d.); eluents are 290 and 650 mmol L^{-1} potassium phosphate buffers at pH 7.4; flow rate: 1.0 mL min^{-1}; sample is the 1.5 mL concentrate of serum protein-VLDL CCC fraction containing 41.8 mg of total proteins (CCC-fr. 2).

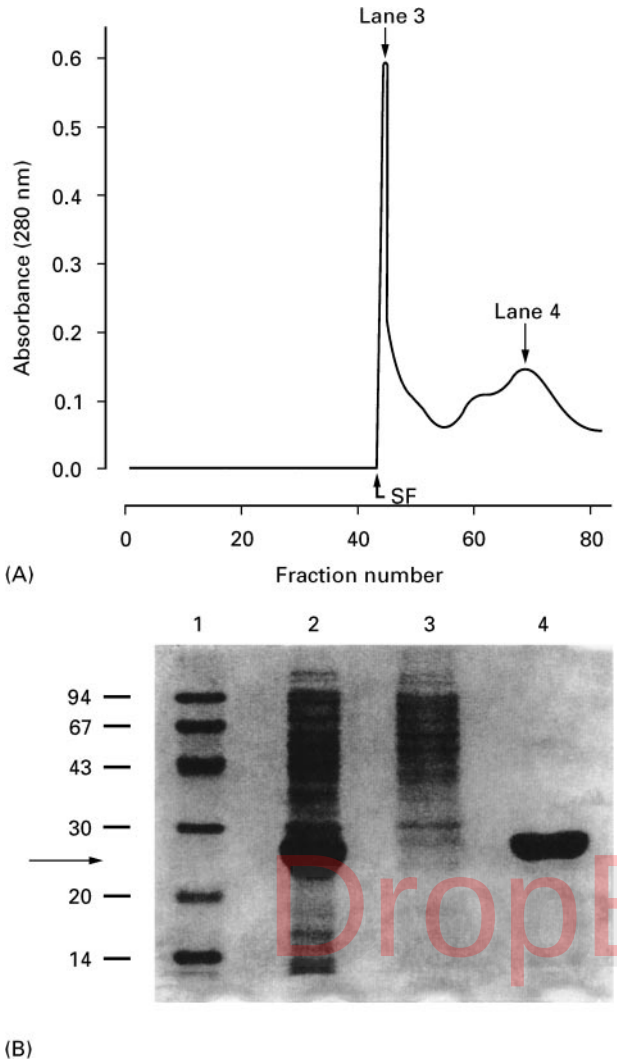

Figure 8 Countercurrent chromatographic purification of purine nucleoside phosphorylase (PNP) from crude extract of *Escherichia coli* (A) and SDS gel electrophoresis profile of CCC fractions (B). Experimental conditions: column is a 2.6 mm i.d. PTFE multilayer coil $\times 2$, $\beta = 0.25$–0.60, 250 mL capacity; sample consists of crude PNP in 10 mL solvent; solvent system consists of 16% (w/w) PEG 1000, 6.25% (w/w) K_2HPO_4 6.25% (w/w) KH_2PO_4 (pH 6.8); mobile phase is the upper phase; flow rate: 0.5 mL min^{-1}; revolution: 750 rpm; SF, solvent front. Lane 1, molecular weights markers; Lane 2, crude extract; Lane 3, HSCCC fraction 46 (solvent front); Lane 4, HSCCC fraction 71 (213 mL).

in HA-fr. 1, HA-fr. 2 and HA-fr. 4, respectively. From the results of SDS-PAGE of the hydroxyapatite fractions, HA-frs. 1, 2 and 4 are free from serum proteins and HA-fr. 3 contained only serum proteins.

Purification of Recombinant Enzymes from Crude *Escherichia coli* Lysate

The capability of the XLL cross-axis CPC was further examined in the purification of some recombinant enzymes from a crude extract of *Escherichia coli* lysate. The polymer-phase system used was 16% (w/w) PEG 1000, 6.25% (w/w) monobasic and 6.25% (w/w) dibasic potassium phosphate (pH 6.8). The phosphate-rich lower phase was used as the stationary phase. About 1.0 mL of crude lysate containing purine nucleoside phosphorylase (PNP) in 10 mL of the above solvent system was loaded into the multilayer coil and eluted with the PEG-rich upper phase at a flow rate of 0.5 mL min^{-1}. **Figure 8A** shows the chromatogram of crude PNP lysate obtained. A 3 mL volume was collected in each fraction. The solvent front emerged at the 46th fraction (138 mL retention volume) and purified PNP was obtained from fractions 65–80 (195–240 mL). **Figure 8B** shows the 12% SDS gel electrophoresis patterns of the CCC fractions obtained from the crude PNP lysate. Gel electrophoresis clearly demonstrates that PNP in the crude *E. coli* lysate was highly purified by CCC via a single pass through the column.

Purification of recombinant uridine phosphorylase (UrdPase) from *E. coli* lysate has been performed similarly, as shown in **Figure 9**. The polymer phase system was the same as that used for the purification of recombinant PNP described above. About 2.0 mL of the crude lysate in 4 mL of the solvent, 1 mL of upper phase and 3 mL of lower phase containing 16% PEG 1000 and 12.5% potassium phosphate, was loaded on the column and eluted with the PEG-rich upper phase at 0.5 mL min^{-1}. In Figure 9, protein concentration in the eluted fractions (solid line) is plotted against the retention volume. The chromatogram shows four protein peaks. Most of the protein mass was eluted immediately after the solvent front in fractions 35–55 (105–165 mL), whereas the enzyme activity of the UrdPase coincides with the fourth protein peak corresponding to fractions 75–95 (230–285 mL). These results indicate that recombinant UrdPase can be highly purified from the crude *E. coli* lysate in a one-step operation within 10 h by the XLL cross-axis CPC.

Purification of Lactic Acid Dehydrogenase from Bovine Heart Crude Extract

CCC has been applied to the purification of lactic acid dehydrogenase (LDH) from a crude bovine heart filtrate using the XL cross-axis CPC. The separation was performed with a polymer-phase system, composed of 16% (w/w) PEG 1000, 12.5% (w/w) potassium phosphate at pH 7.3.

Figure 10A shows chromatograms of the bovine heart crude extract obtained, where the PEG-rich upper phase was used as the stationary phase. The separation was performed at 500 rpm at a flow rate of 1.0 mL min^{-1} using the phosphate-rich lower phase as the mobile phase. The enzymatic activity of LDH was detected between the second and third

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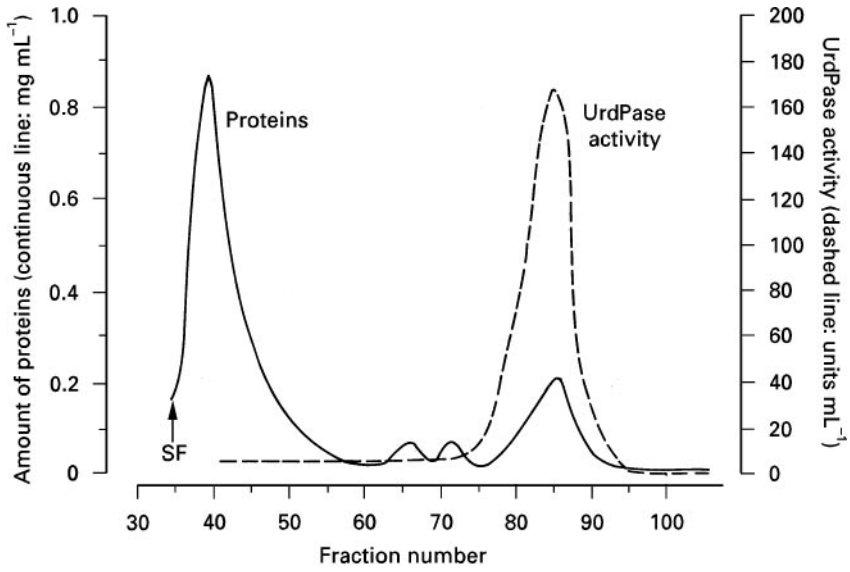

Figure 9 Countercurrent chromatographic purification of uridine phosphorylase (UrdPase) from crude *Escherichia coli* lysate. Experimental conditions: column is a 2.6 mm i.d. PTFE multilayer coil × 2, $\beta = 0.25–0.60$, 250 mL capacity; sample consists of 2 mL crude UrdPase in 4 mL solvent; solvent system consists of 16% (w/w) PEG 1000, 6.25% (w/w) K_2HPO_4 and 6.25% (w/w) KH_2PO_4 (pH 6.8); mobile phase is the lower phase; flow rate: 0.5 mL min^{-1}; revolution: 750 rpm; SF, solvent front.

peaks. These fractions were analysed by 12% (w/v) SDS-PAGE with Coomassie Brilliant Blue staining (Figure 10B), indicating that the LDH is actually contained in 30 mL of eluent (fractions 140–170 mL) without detectable contamination from other proteins. The traditional techniques used for purification of LDH require several steps, including precipitation with ammonium sulfate, centrifugation and dialysis; hence they are very tedious and time-consuming. By combined use of the XL cross-axis CPC and the aqueous polymer-phase system described above, LDH is purified within 3 h.

These results show that, with relatively simple manipulation of several parameters (buffer, polymer molecular mass, rotation speed), CCC is well suited to the rapid purification of enzymes from very crude

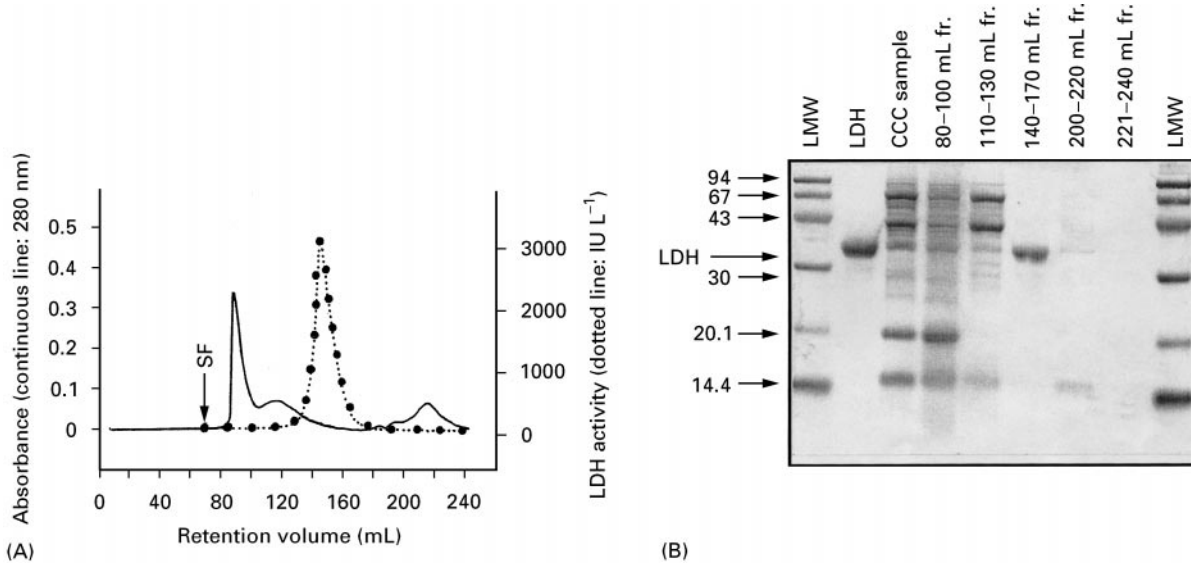

Figure 10 (See Colour Plate 116) (A) Countercurrent chromatography of bovine heart homogenate and (B) SDS-PAGE profile of the fractions. Experimental conditions: column is a 2.6 mm i.d. PTFE multilayer coil × 2, $\beta = 0.50–0.60$, 165 mL capacity; sample is a mixture of 3 mL bovine heart crude extract, 3 mL solvent system (1.5 mL each phase); solvent system consists of 16% (w/w) PEG 1000 12.5% (w/w) potassium phosphate (pH 7.3); mobile phase is the lower phase; flow rate: 2.0 mL min^{-1}; revolution: 500 rpm; SF, solvent front. LMW, low molecular weight protein markers.

tissue extracts. Because of the protective effect of a high concentration of polymers and potassium phosphate, the native structure of the proteins is preserved at room temperature during separation, and the support-free partitioning eliminates sample loss and deactivation of enzymes which is often caused by using the solid support in conventional chromatography. We expect that these merits of the method will apply in the purification of other enzymes.

Conclusion

The capability of the cross-axis CPCs for performing CCC has been demonstrated in the separation and purification of proteins. The unique feature of the apparatus is that it provides sufficient retention of the stationary phase for viscous, low interfacial tension polar solvent systems, such as aqueous-aqueous polymer phase systems. Consequently, the method can be utilized for the fractionation of a wide variety of proteins without adsorptive sample loss and denaturation of proteins caused by the solid support. The CCC method may be further extended to the purification and fractionation of other biopolymers.

See Colour Plate 116.

See also: **II/Chromatography:** Countercurrent Chromatography and High-Speed Countercurrent Chromatography: Instrumentation. **Chromatography: Liquid:** Countercurrent Liquid Chromatography. **Appendix 1: Essential Guides for Isolation/Purification of Enzymes and Proteins; Essential Guides for Isolation/Purification of Immunoglobulins.**

Further Reading

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Ion Exchange

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Introduction

Proteins are polymers of amino acids, the so-called 'building blocks of nature' and are found in all living matter be it of animal, microbial or vegetable origin. By their very structure proteins have an electrical charge and can therefore be fractionated by ion exchange processes. This paper briefly reviews the principles underlying protein purification by ion exchange and addresses some of the process issues associated with their purification.

Proteins

As living cells reproduce, genetic material is passed from parent cells to daughter cells in the form of DNA. DNA is a template coding for the various proteins required for the developing organism. As the organism grows, cells differentiate to form the various organs of the mature organism. Each cell has the capability to express every single protein of the organism, but in life only a small fraction of proteins are expressed. For example muscle cells produce actin and myosin to facilitate movement, the pancreas produces chymotrypsinogen and trypsinogen to facilitate digestion and lymphocytes are responsible for the expression of immunoglobulins which provide immunity from infection and disease.